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APPLICANTS:	Kong et al.	EXAMINER:	Strzelecka
SERIAL NO.:	10/665,633	GROUP:	1637
FILED:	September 19, 2003		
FOR:	Helicase-Dependent Amplification of Nucleic Acids		

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DECLARATION UNDER 37 C.F.R. §1.132

I hereby declare that:

1. My name is Dr Huimin Kong, President of BioHelix Corp., exclusive licensee for the above-referenced patent application. I am an inventor on the present application. My resume is attached.

2. Helicase dependent amplification requires helicase. In the absence of helicase, no amplification could be detected by gel electrophoresis. We performed numerous experiments that confirm the requirement for helicase. A peer reviewed paper is attached, where Figure 2 shows this effect. (*EMBO rep* 5, 795-800). The significance of this finding is underscored by news reports on this helicase dependent amplification in Nature as well as in popular science magazines such as the New Scientist and The Scientist.

3. I further declare under penalty of perjury pursuant to laws of the United States of America that the foregoing is true and correct and that the Declaration was executed by me on:

Huimin Kong

Dr. Huimin Kong Date: January 25, 2007

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel and other significant contributors.
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NAME	POSITION TITLE
Huimin Kong	President
eRA COMMONS USER NAME biohelix	

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
Fudan University, Shanghai, China	B.S.	1984	Genetics
Fudan University, Shanghai, China	M.S.	1987	Microbiology
Boston University	Ph.D.	1998	Biology

Please refer to the application instructions in order to complete sections A, B, and C of the Biographical Sketch.

A. Positions and Honors.

1987-1989 Research fellow, Institute of Genetics, Fudan University
1989-1992 Research associate, New England Biolabs Inc.
1992-1998 Research scientist, New England Biolabs, Inc.
1998-2001 Staff scientist, New England Biolabs, Inc.
2001-2004 Senior scientist, New England Biolabs, Inc.
2004- President, BioHelix Corporation

B. Selected peer-reviewed publications (in chronological order).

1. Kong, H. and Chen, Z. (1987) Isolation and identification of restriction endonuclease *Bst*FI. *Nucleic Acids Res.* 15, 7205.
2. Chen, Z. and Kong, H. (1988) Isolation and characterization of restriction endonuclease *Bst*YI from *Bacillus stearothermophilus*. *FEBS LETTERS* 234, 169-171.
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C. Research Support.

Ongoing Research Support

2 R44 GM071155-02 Kong (PI) 4//01/2005 – 3/31/2007
NIH/NIGMS
"Kilo-base Range Isothermal DNA Amplification"
Overall goals: To develop and commercialize a novel isothermal DNA amplification method capable of amplifying long range (kilo-base) DNA fragments.
Role: PI

1R43AI066487-01 Kong (PI) 7//01/2005 – 6/30/2007
NIH/NIAID
"Development of a helicase-based rapid DNA diagnostic system for Biodefense"
Overall goals: To further improve the performance of HDA by overcoming technical barriers limiting the current detection sensitivity and speed for both DNA and RNA targets.
Role: PI

Completed Research Support

1 R43 GM073402-01 Kong (PI) 03/14/2005 – 03/31/2006
NIH/NIGMS
"True-Isothermal Plasmid Amplification System"
Overall goals: To develop a novel *in vitro* plasmid DNA amplification method and kits.
Role: PI

BAA 04-03; Full Proposal #45 Kong (PI) 4/15/2005 – 4/14/2006
The Department of Homeland Security
"A helicase-based next generation polymerase chain reaction system"
Overall goals: To develop HDA-based field deployable hand-held nucleic acid diagnostic devices to detect biothreat organisms such as *Bacillus anthracis* in the field.
Role: PI

1 R43 GM071155-01 Kong (PI) 05/01/04 – 10/31/04
NIH/NIGMS
Kilo-base Range Isothermal DNA Amplification
Role: PI

2 R43 GM60057-03 Kong (PI) 09/01/01 – 8/31/03
NIH/NIGMS
Engineering New Nicking Endonucleases.
Role: PI

1 R43 GM60057-01 Kong (PI) 07/01/99 – 12/31/99
NIH/NIGMS
Engineering New Nicking Endonucleases.
Role: PI

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Helicase-dependent isothermal DNA amplification

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New England Biolabs, Beverly, Massachusetts, USA

Polymerase chain reaction is the most widely used method for *in vitro* DNA amplification. However, it requires thermocycling to separate two DNA strands. *In vivo*, DNA is replicated by DNA polymerases with various accessory proteins, including a DNA helicase that acts to separate duplex DNA. We have devised a new *in vitro* isothermal DNA amplification method by mimicking this *in vivo* mechanism. Helicase-dependent amplification (HDA) utilizes a DNA helicase to generate single-stranded templates for primer hybridization and subsequent primer extension by a DNA polymerase. HDA does not require thermocycling. In addition, it offers several advantages over other isothermal DNA amplification methods by having a simple reaction scheme and being a true isothermal reaction that can be performed at one temperature for the entire process. These properties offer a great potential for the development of simple portable DNA diagnostic devices to be used in the field and at the point-of-care.

Keywords: DNA amplification; isothermal; helicase; DNA polymerase; UvrD
EMBO reports (2004) 5, 795–800. doi:10.1038/sj.embor.7400200

INTRODUCTION

The polymerase chain reaction (PCR) revolutionized our capabilities to do biological research, and it has been widely used in biomedical research and disease diagnostics (Saiki *et al*, 1988). Hand-held diagnostic devices, which can be used to detect pathogens in the field and at point-of-care, are demanded currently. However, the need for power-hungry thermocycling limits PCR application in such a situation. Several isothermal target amplification methods have been developed (Andras *et al*, 2001). Strand-displacement amplification (SDA) combines the ability of a restriction endonuclease to nick the unmodified strand of its target DNA and the action of an exonuclease-deficient DNA polymerase to extend the 3′ end at the nick and displace the downstream DNA strand (Walker *et al*, 1992). Transcription-mediated amplification (TMA) uses an RNA polymerase to make RNA from a promoter engineered in the primer region, a reverse transcriptase to produce complementary DNA from the RNA templates and RNase H to remove the RNA from cDNA (Guatelli *et al*, 1990). In the rolling circle amplification (RCA), a DNA polymerase extends a primer on a circular template, generating tandemly linked copies of the complementary sequence of the template (Fire & Xu, 1995). However, these isothermal nucleic acid amplification methods also have their limitations. Most of them have complicated reaction schemes. In addition, they are incapable of amplifying DNA targets of sufficient length to be useful for many research and diagnostic applications.

In living organisms, a DNA helicase is used to separate two complementary DNA strands during DNA replication (Kornberg & Baker, 1992). We have devised a new isothermal DNA amplification technology, helicase-dependent amplification (HDA), by mimicking nature. HDA uses a DNA helicase to separate double-stranded DNA (dsDNA) and generate single-stranded templates for primer hybridization and subsequent extension. As the DNA helicase unwinds dsDNA enzymatically, the initial heat denaturation and subsequent thermocycling steps required by PCR can all be omitted. Thus, HDA provides a simple DNA amplification scheme: one temperature from the beginning to the end of the reaction. In this study, we present the *Escherichia coli* UvrD-based HDA system, which can achieve over a million-fold amplification.

RESULTS

HDA design

The fundamental reaction scheme of HDA is shown in Fig 1. In this system, strands of duplex DNA are separated by a DNA helicase and coated by single-stranded DNA (ssDNA)-binding proteins (SSBs; Fig 1, step 1). Two sequence-specific primers hybridize to each border of the target DNA (Fig 1, step 2). DNA polymerases extend the primers annealed to the templates to produce a dsDNA (Fig 1, step 3). The two newly synthesized dsDNA products are then used as substrates by DNA helicases, entering the next round of the reaction (Fig 1, step 4). Thus, a simultaneous chain reaction proceeds resulting in exponential amplification of the selected target sequence.

E. coli UvrD helicase was chosen as the DNA helicase for our first HDA system because it can unwind blunt-ended DNA fragments (Runyon & Lohman, 1989). The SSB in the HDA reaction is either bacteriophage T4 gene 32 protein (Casas-Finet & Karpel, 1993) or RB 49 gene 32 protein (Desplats *et al*, 2002).

Amplification of a target sequence from plasmid DNA

Two M13/pUC19 universal primers (1224 and 1233) were used in an HDA reaction to amplify selectively a 110 base pair (bp)

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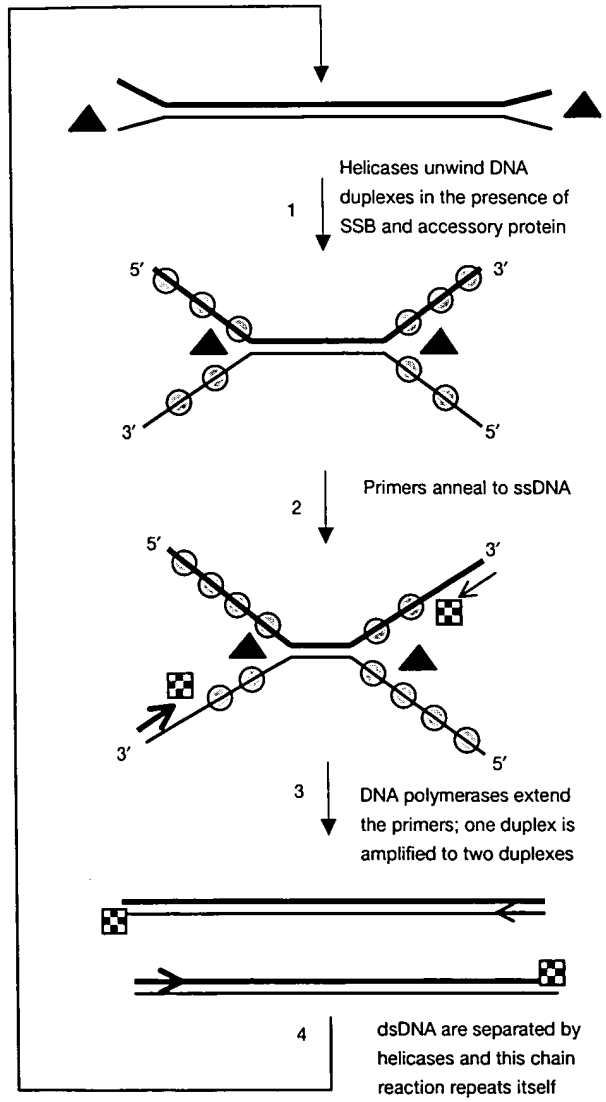

Fig 1 | Schematic diagram of HDA. Two complementary DNA strands are shown as two lines: the thick one is the top strand and the thin one is the bottom strand. 1: A helicase (black triangle) separates the two complementary DNA strands, which are bound by SSB (grey circles). 2: Primers (lines with arrow heads) hybridize to the target region on the ssDNA template. 3: A DNA polymerase (squares with mosaic patterns) extends the primers hybridized on the template DNA. 4: Amplified products enter the next round of amplification.

target sequence from a derivative of pUC19 plasmid. In a first step, substrate DNA was mixed with the primers for heat denaturation and subsequent annealing. The component B mixture containing key enzymes, such as *E. coli* UvrD helicase plus its accessory protein MutL, phage T4 gene 32 protein and the exo$^-$ Klenow fragment of DNA polymerase I, was then added into component A. After a 1 hr incubation period at 37 °C, a 110-bp amplification product was observed on a 2% agarose gel (Fig 2, lane 1). Sequencing results confirmed that it matched the target DNA sequence.

To determine the essential elements in the HDA reaction, each key component was omitted from the reaction. In the absence of

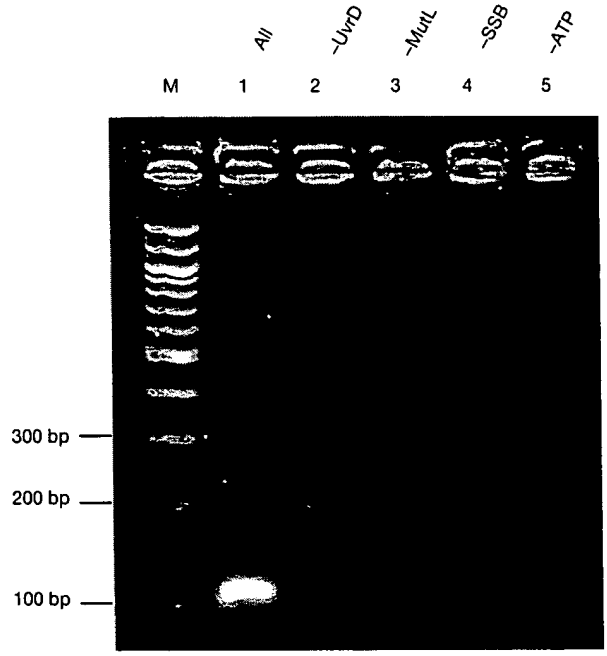

Fig 2 | Electrophoresis of HDA products amplified from plasmid DNA. A two-step HDA reaction, with a 1 h incubation at 37 °C, was performed in the presence of all components (lane 1) including a pUC19-derived plasmid DNA (0.035 pmol), primer-1224 (10 pmol) and primer-1233 (10 pmol), UvrD helicase (100 ng), MutL (400 ng), T4 gene 32 protein (4.5 μg), ATP (0.15 μmol) and exo$^-$ Klenow polymerase (5 U). HDA products in the absence of UvrD helicase (lane 2), accessory protein MutL (lane 3), T4 gene 32 protein (lane 4) or ATP (lane 5) are shown. M: 100-bp DNA ladder.

UvrD helicase, no amplification was observed (Fig 2, lane 2), confirming that helicase is required for the amplification. In the absence of accessory protein MutL, no amplification product was observed (Fig 2, lane 3), suggesting that UvrD helicase mediated-amplification requires MutL. *In vivo*, MutL, the master coordinator of mismatch repair, recruits UvrD helicase to unwind the DNA strand containing the replication error (Lahue *et al*, 1989). MutL stimulates UvrD helicase activity more than tenfold by loading it onto the DNA substrate (Mechanic *et al*, 2000). In the absence of T4 gene 32 protein, again no amplification product was observed (Fig 2A, lane 4), indicating that SSB is required in this reaction, probably to prevent reassociation of the complementary ssDNA templates at 37 °C. In the absence of ATP, no amplification product was detected, indicating that the helicase cofactor is essential for HDA. Target sequences up to 400 bp can be efficiently amplified from plasmid DNA, beyond which the yield drops markedly (data not shown).

Amplification of target sequences from genomic DNA

To test whether HDA can be used to amplify a specific sequence from more complex DNA samples, such as bacterial genomic DNA, the *E. coli* UvrD-based HDA system was used to amplify a 123-bp fragment from an oral pathogen, *Treponema denticola*. A restriction endonuclease gene encoding a homologue of *earIR* (GenBank accession number: TDE0228) was chosen as the target gene. The amplification power of the current HDA system was

also determined by decreasing the amount of *T. denticola* genomic DNA. The amount of template was varied from 10^7 to 10^3 copies of the *T. denticola* genome. In general, the intensities of the HDA product decreased as the initial copy number was lowered (Fig 3A). With 10^3 copies of initial target, about 10 ng of products were generated, which corresponds to 10^{10} molecules of the 123-bp fragment. Thus, the current HDA system described here is capable of achieving over ten million-fold amplification. The negative control, containing no *T. denticola* genomic DNA, showed no trace of amplified products, proving the specificity and reliability of HDA.

In addition to *T. denticola*, the *E. coli* UvrD-based HDA system can amplify target sequences from various genomic DNAs isolated from *Helicobacter pylori, E. coli, Neisseria gonorrhoeae, Brugia malayi* and human cells (data not shown).

One temperature HDA

As helicases are able to unwind duplex DNA enzymatically, we tested whether the entire HDA reaction could be carried out at one temperature without prior heat denaturation. Another region (102 bp) of the *earIR* homologue gene was chosen as target. Component B was added to A either immediately or after a denaturation step. The yield of the one-step HDA amplification was about 40–60% of the two-step HDA reaction. Nevertheless, enough product is generated to be detected (Fig 3B). This demonstrates that HDA is able to amplify a target sequence from bacterial genomic DNA at one temperature for the entire process.

Amplification of a target sequence from *T. denticola* cells

To test whether HDA can be used on crude samples, the reaction was carried out directly on bacterial cells. A 111-bp sequence within *T. denticola* glycogen phosphorylase gene (GenBank accession number: TDE2411) was chosen as target. A specific product was obtained when using 10^7 to 10^4 cells as template (Fig 3C). As the initial cell number was lowered, the intensity of the HDA-specific product decreased and other products of lower molecular weight were observed. These products are non-target specific as they could also be detected for the negative control. They result from a nonspecific amplification and are most probably derivates of primer-dimers. Primer-dimers can be generated by the HDA reaction when the template amount is very low; they also occur in the PCR reaction (Brownie *et al*, 1997). Nevertheless, the negative control allows us to distinguish the target-specific from the non-target-specific products. The current HDA system can work on crude samples, such as whole bacterial cells with only a tenfold loss of sensitivity compared with the purified genomic DNA (Fig 3B).

Detection of *B. malayi* DNA in blood

To test the possibility of using HDA on real samples, a pathogen's DNA sequence was amplified in the presence of human blood. A 99-bp fragment of the *Hha*I repeat of the filarial parasite *B. malayi* was chosen as target. First reported to comprise 10–12% (McReynolds *et al*, 1986), and then 1% of the *Brugia* genome (Ghedin *et al*, 2004), this highly repeated sequence became a target of choice for the detection of *B. malayi* (Rao *et al*, 2002). Decreasing amounts of *B. malayi* genomic DNA were added to human blood samples. After extraction and dialysis, the samples were used as templates for HDA reactions. A specific product was detected for samples containing as low as 5 pg of *B. malayi* DNA, which corresponds to 500 copies of the genome (Fig 4). These results demonstrate the feasibility of using HDA to detect a pathogen in a real sample.

Real-time HDA

We have developed a real-time detection system using a LUX™ primer specific to the earIR homologue gene in *T. denticola*. Two identical HDA reactions (curves 1 and 2) along with a negative control (curve 3) were performed (Fig 5A). After 35 min, product accumulation generated a typical sigmoid curve. A semilogarithmic plot of the increase in fluorescence in the early phase of the reaction revealed an initial first-order reaction with a rate of

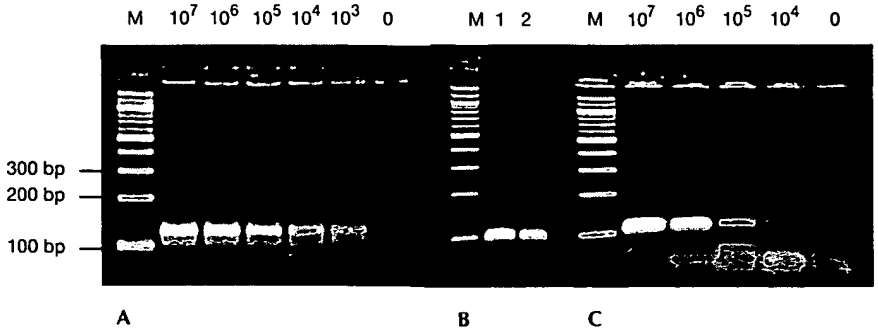

Fig 3 | Electrophoresis of HDA products amplified from bacterial genomic DNA. (A) Amplification of a 123-bp target sequence from *T. denticola* genomic DNA. A two-step HDA reaction, with a 3 h incubation at 37 °C, was performed in the presence of primer Ea136for (20 pmol) and primer Ea136rev (20 pmol), UvrD helicase (100 ng), MutL (800 ng), T4 gene 32 protein (4.5 μg), ATP (0.15 μmol) and exo⁻ Klenow polymerase (5 U). The copy number of the single *T. denticola* chromosome initially present in each HDA reaction is shown above each lane. (B) Amplification of a 102-bp target sequence with (lane 1) or without (lane 2) heat denaturation at 95 °C, before a 2 h incubation at 37 °C. The reaction was set up as described in (A), except that the primers used were Ea1for and Ea81rev. (C) Amplification of a 111-bp target sequence from *T. denticola* cells. A two-step HDA reaction, with a 10 min incubation at 95 °C and 2 h incubation at 37 °C, was performed. The reaction was set up as described in (A), except that the primers used were Gp98for and Gp188rev and 5.8 μg of RB49 gene 32 protein replaced 4.5 μg of T4 gene 32 protein. Frozen *T. denticola* cells were diluted in water and the initial amount present in each HDA reaction is shown above each lane. M: 100-bp DNA ladder.

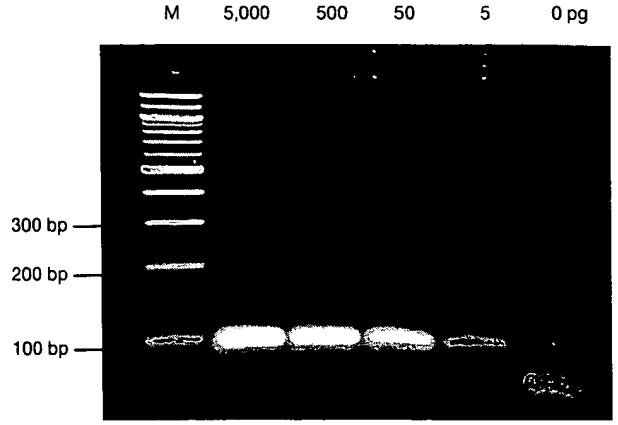

Fig 4 | Electrophoresis of 99-bp HDA products amplified from *B. malayi* genomic DNA in human blood samples. A 0.1–1,000 ng portion of *B. malayi* genomic DNA was added to 200 µl of human blood samples. After processing, 1 µl of each sample was used as template for HDA reactions. The amount of *B. malayi* genomic DNA initially present in each HDA reaction is shown above each lane. A two-step HDA reaction, with a 2 h incubation at 37 °C, was performed. M: 100-bp DNA ladder.

amplification (V) of 0.23 RFU/min, which corresponds to a doubling time of 3 min (Fig 5B). Following the log-linear phase, the reaction slowed, entering a transition phase (between 45 and 80 min), eventually reaching the plateau phase (Fig 5A). Curves 1 and 2 derived from two identical reactions were very similar, suggesting that the real-time HDA reaction has a good reproducibility. In the negative control, the fluorescent signal remained below the T_t (time of threshold) line (Fig 5A, curve 3) and no amplified DNA was observed on the agarose gel (Fig 5C, lane 3).

DISCUSSION

In this study, we report a new isothermal DNA amplification technique, named HDA. It has a significant advantage over PCR in that it eliminates the need for an expensive and power-hungry thermocycler. HDA also offers several advantages over existing isothermal DNA amplification methods. First, it has a simple reaction scheme, in which a target sequence can be amplified by two flanking primers, similar to PCR (Fig 1). In contrast, other isothermal DNA amplification techniques have complicated reaction mechanisms and experimental designs. For example, SDA uses four primers to generate initial amplicons and modified deoxynucleotides to provide strand-specific nicking (Walker *et al*, 1992). TMA needs three different enzymatic steps (transcription/cDNA synthesis/RNA degradation) to accomplish an isothermal RNA amplification (Guatelli *et al*, 1990). This complexity and the inefficiency in amplifying long targets limit their use in biomedical research. As a result, these isothermal amplification techniques are primarily used in specifically designed diagnostic assays, and PCR remains the only protocol used by researchers to amplify specific targets of DNA.

Second, HDA is a true isothermal DNA amplification method. As DNA helicase can melt double-stranded target DNA at the beginning of the reaction, the entire HDA reaction can be performed at one temperature (Fig 3B). In contrast, other isothermal methods, such as SDA, still need an initial heat denaturation step at a high temperature followed by amplification at a lower temperature (Walker *et al*, 1992).

Third, HDA is at its early development stage. The current UvrD system can achieve over a million-fold amplification. A pathogen genomic DNA can even be detected in a human blood sample. This demonstrates that HDA can be performed on crude samples and has the potential to be used as a diagnostic tool. *E. coli* UvrD helicase, a repair helicase, was chosen as our model system because it is a well-studied helicase and it unwinds blunt-end substrates. However, its speed (20 bp/s) and processivity (less than 100 bp per binding) are limited (Ali *et al*, 1999). MutL can stimulate UvrD unwinding activity but fails to increase its processivity (Mechanic *et al*, 2000). This may explain as to why the current UvrD HDA system is inefficient at amplifying long target sequences. The performance of an HDA system may be further improved by testing different helicases. DNA helicases are found in all organisms and participate in major cellular DNA metabolisms including replication, repair and recombination (Kornberg & Baker, 1992; Caruthers & McKay, 2002). In a recent experiment, we were able to amplify a 2.5-kb target from a plasmid DNA by using a processive replicative helicase, T7 gene 4 protein (Y. Xu and H. Kong, unpublished data), which unwinds DNA at a rate of 300 bp/s and with high processivity (Kornberg & Baker, 1992).

Optimization of current HDA systems involves identifying rate-limiting steps. In the HDA reaction, the unwinding, primer-annealing and extension steps must be coordinated. One of the rate-limiting steps could be the coordination between the helicase and the DNA polymerase. The exo$^-$ Klenow fragment can be substituted by other polymerases such as T7 sequenase (USB) or Klenow fragment, but none of these polymerases improved the reaction (data not shown). A DNA polymerase, which can move with the DNA helicase in a coordinated way, would be an ideal combination. This kind of coordination can be found at the *in vivo* replication fork where DNA polymerase III interacts with the DnaB helicase (Kornberg & Baker, 1992). One way to achieve this kind of coordination is to use a helicase/polymerase pair that works together naturally. Another rate-limiting step could be the interaction between SSB and DNA. The essential role of SSB in the HDA reaction is probably to prevent the reassociation of the separated DNA strands. Indeed, no DNA amplification was observed in the absence of SSB. Both T4 gene 32 protein (Lohman, 1984) and RB49 gene 32 protein (Desplats *et al*, 2002) can efficiently support the HDA reaction. They can be substituted by *E. coli* SSB (Bujalowski & Lohman, 1989) or T7 gene 2.5 SSB (Nakai & Richardson, 1988), but the yield of amplification is lower (data not shown).

Future experiments will be directed towards improving the efficiency of HDA by testing different helicases/polymerases and by optimizing the existing HDA systems by varying the ratio and concentration of each key component. Indeed, the concentration of each protein in an HDA reaction has significant effects on the outcome of the reaction. Deviation from the optimal concentration results in a decrease in the yield and, eventually, failure of the amplification process. The simplicity and true isothermal nature of the HDA platform offer great potential for the development of hand-held DNA diagnostic devices that could be used to detect pathogens at point-of-care or in the field.

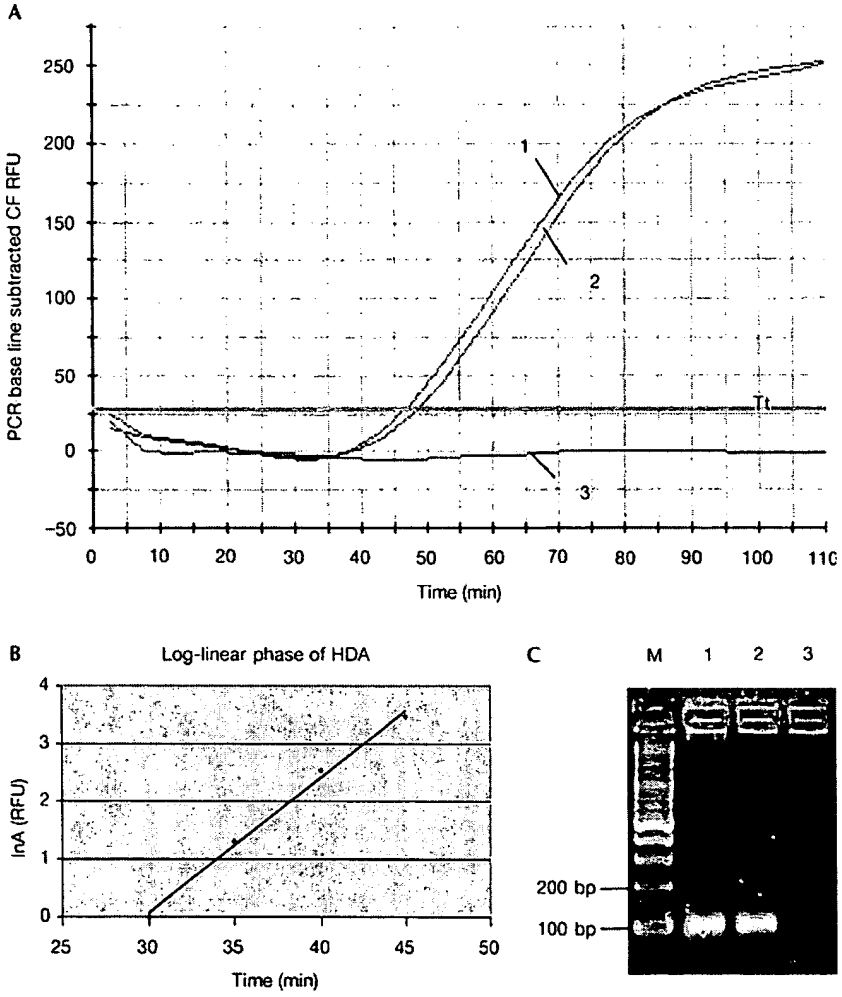

Fig 5 | Real-time HDA. A 97-bp fragment from *T. denticola* genomic DNA was amplified using a LUX primer. (A) Amplification products were detected in real time by measuring fluorescent signals (relative fluorescence unit (RFU)). Curves 1 and 2: two identical reactions performed on 10 ng of genomic DNA. Curve 3: reaction similar to curves 1 and 2 but without any genomic DNA (negative control). (B) Re-plotting data points corresponding to the early phase of the reaction, shown in (A), as the log of amplified products against time. Doubling time was calculated using the formula $t_{1/2} = \ln 2/V$. (C) Electrophoresis of final amplified products. M: 100-bp DNA ladder.

METHODS

Material. T4 gene 32 protein was purchased from Roche Applied Science. Adenosine 5′-triphosphate (ATP) was purchased from Amersham Biosciences. Primer-175-LUX was purchased from Invitrogen. All other enzymes and reagents including exo⁻ Klenow fragment, pTYB1, pTYB3, pTXB1, dNTPs and oligodeoxynucleotides were from New England Biolabs.

Cloning and purification of UvrD helicase and MutL from *E. coli*. *uvrD* (Swissprot accession number: P03018) and *mutL* (Swissprot accession number: P23367) genes were amplified from *E. coli* K12 genomic DNA using PCR and cloned into the *Nco*I and *Sap*I sites of pTYB3 and *Nde*I and *Sap*I sites of pTYB1, respectively, to construct C-terminal fusions with a self-cleavable affinity tag (Impact™ system, NEB) (Chong *et al*, 1998). See supplementary information online for details on purification.

Cloning and purification of gene protein 32 from bacteriophage RB 49. Gene 32 (GenBank accession number: NP_891812) was amplified from RB49 genomic DNA using PCR and cloned into the *Nde*I and *Sap*I sites of pTXB1 to construct C-terminal fusions with a self-cleavable affinity tag (Impact™ system, NEB) (Chong *et al*, 1998). See supplementary information online for details on purification.

HDA reactions for amplifying target sequence. Two HDA buffers were prepared. The 10 × HDA buffer A contains 350 mM Tris-acetate (pH 7.5) and 100 mM dithiothreitol and the 10 × HDA buffer B contains 10 mM Tris-acetate (pH 7.5), 1 mg/ml bovine serum albumin and 100 mM magnesium acetate. HDA reaction component A (30 μl) was prepared by combining 5 μl of 10 × HDA buffer A, template (plasmid DNA, genomic DNA, cells, processed human blood sample (see supplementary information online for information on the preparation of the reconstituted human blood sample)), 10–20 pmol of each target-specific primer (see supplementary information online for details on the HDA primers), 20 nmol dNTPs and dH_2O. The reaction component A was heated for 2–10 min at 95 °C to denature the template and 1–4 min at 37 °C. Reaction component B (20 μl) was freshly

prepared by mixing 5 µl of 10 × HDA buffer B, 150 nmol ATP, 5 U exo⁻ Klenow fragment, 100 ng UvrD helicase, 400–800 ng MutL protein, 4.5 µg T4 gp32 or 5.8 µg RB49 gp32, and dH_2O. Component B was then added to component A. The reaction was continued for 1–3 h at 37 °C and was then terminated by addition of 12.5 µl of stop buffer (0.1% sodium dodecyl suphate, 50 mM Na_2EDTA, 15% Ficoll and 0.2% orange G). Reaction products were analysed on a 2% GPG LMP agarose gel containing ethidium bromide. The HDA reaction without heat denaturation was set up by combining all the elements mentioned above in the same tube and incubating directly for 2 h at 37 °C. To monitor HDA in real time, fluorescent primers were used (primer-175-LUX). The amplification products were detected by measuring fluorescent signals at 490 nM at 5 min intervals using an iCyler (Bio-Rad).

Supplementary information is available at *EMBO reports* online (http://www.emboreports.org).

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research highlights

Chemistry

Green fuels pick up the pace

Green Chem. 6, doi:10.1039/b404883k (2004)

Biodiesel is an alternative fuel derived from fatty acids in vegetable oils or animal fats. Because it contains almost no sulphur, it burns more cleanly than conventional diesel. However, the chemical reaction that breaks down the fatty acids into fuel relies on an aqueous alkali catalyst such as sodium hydroxide, making the product difficult to separate from the reaction mixture.

Robert S. Watkins *et al.* now unveil a solid catalyst for this reaction that works just as well as a conventional catalyst, yet allows much easier separation of products. Their experiments used starting materials that contain shorter carbon chains than those found in vegetable oils, as a simpler model system for screening potential catalysts.

The authors achieved a 100% yield of the product using a calcium oxide catalyst loaded with 1.23% (by weight) lithium nitrate. The catalyst could be easily filtered from the reaction mixture, and recycled with minimal loss of activity. This could open up the possibility of synthesizing biodiesel as a continuous process, rather than in batches. **Mark Peplow**

Plant biology

Stretching a point

Plant Physiol. doi:10.1104/pp.104.041483 (2004)

The growth of a pollen tube from a pollen grain depends on fluxes of metal ions. In particular, a calcium gradient, with highest concentrations in the tip, is needed to sustain tube elongation. Rajiv Dutta and Kenneth R. Robinson have studied events in the Easter lily, *Lilium longiflorum*, and have identified an ion channel that is responsible for calcium influx into the growing tube, and which is activated by stretching of the cell membrane.

The lily pollen tube grows in cycles of elongation every 40 to 60 seconds, accompanied by oscillations in the concentrations of various ions. Using electrophysiological measurements, Dutta and Robinson detected a spontaneously active potassium channel in the membrane of pollen grains, as well as potassium and calcium channels that were activated when the membrane was stretched by the measuring pipette. Only stretch-activated calcium channels were detected in pollen-tube tips. Blockade of this channel inhibited tube growth.

The increase in intracellular calcium occurs slightly after the elongation phase of pollen-tube growth, in line with the idea that a stretch-activated channel is involved. This process would also position calcium influx at the point of maximum elongation, providing a feedback loop that could account for the growth oscillations seen in lilies. It remains to be seen how potassium enters the tip. **Christopher Surridge**

Earthquakes

Shock reconstruction

J. Geophys. Res. B 109, doi:10.1029/2003JB002523 (2004)

On 29 June 1170, a powerful earthquake shook the Middle East, developing along a 1,000-km fault that runs from southern Turkey to the Red Sea. Arabic documents of the time record the impact of the quake, but the sources are relatively few and they differ in their assignment of the earthquake's epicentre.

Emanuela Guidoboni and colleagues have delved into contemporary accounts in

Contemporary texts

other languages to build a more complete picture of the event. The timing of the quake between the second and third Crusades (1147–49 and 1189–92) meant that there were people of many nationalities in the region. Among the accounts in Latin are those of William of Tyre (pictured), who wrote of the city of Tripoli being reduced to an "untidy pile of stones"; and of Amalric the First, King of Jerusalem, who informed the King of France by letter that "nearly all the castles and cities situated between Tripoli and Antioch" were destroyed. Michael the Syrian — writing in Syriac, an ancient dialect of Aramaic — records that the northern city of Aleppo was worst hit.

Guidoboni *et al.* used the new data to reconstruct the probable epicentre of the earthquake, in western Syria. They estimate that the quake's magnitude was 7.7, assuming it was a single event: the account by William of Tyre suggests that the region might have been hit twice. **Alison Wright**

Diagnostics

Cool amplification of DNA

EMBO Rep. doi:10.1038/sj.embor.7400200 (2004)

Many diagnostic tests depend on the amplification of a particular DNA sequence for further assays, the main method used being the polymerase chain reaction (PCR). Because the enzyme that copies DNA needs a single-stranded template to make the second strand, the duplex DNA that is generated must be separated by a brief high-temperature pulse in every cycle of amplification. A drawback of this process is that the thermal cycling equipment is both expensive and energy-demanding.

Myriam Vincent *et al.* describe a method for DNA amplification, called helicase-dependent amplification, that doesn't require high temperatures. In their protocol, duplex DNA is separated into single strands by a helicase — a protein that travels along DNA, breaking the bonds between strands. By adding a helicase to a PCR-like reaction, the amplification process can be conducted at constant, lower temperature.

Like PCR, Vincent and colleagues' method can amplify DNA sequences a million-fold. Moreover, the authors suggest how the technique may be improved, making helicase-dependent amplification a potential alternative to standard PCR, particularly where use of a thermocycler is not possible. **Angela K. Eggleston**

Nanotechnology

Nanotube diodes

Appl. Phys. Lett. 85, 145–147 (2004)

Diodes are fundamental components of modern electronics. In the most common type of diode, two different kinds of semiconductor material are joined together — one with an excess of electrons and the other with an excess of positively charged 'holes' for the electrons to fall into. This ensures that current can only flow in one direction through the device.

J. U. Lee *et al.* have made diodes from single-walled carbon nanotubes that measure 0.5–3 nm across and operate efficiently at room temperature. Most semiconductors need a smattering of extra chemical elements to change the number of electrons or holes inside them, but with nanotubes the same effect can be achieved through 'electrostatic doping' — that is, using an external electric field to change the properties of two different parts of the same nanotube. Even better, this means that the field can easily be reversed, changing the preferred direction of current flow.

Lee *et al.* suggest that their nanotube diodes could also function as light-emitting diodes at infrared wavelengths, and could therefore be useful in the development of nanoscale optoelectronic devices. **Mark Peplow**

nature|methods
Techniques for life scientists and chemists

RESEARCH HIGHLIGHTS

Published online: 27 July 2004; | doi:10.1038/nmeth031

DNA CLONING AND AMPLIFICATION

Breaking the Cycle

Michael Eisenstein

A new helicase-based strategy offers a potential alternative to PCR for diagnostic tests, allowing investigators to rapidly amplify specific DNA sequences at a constant reaction temperature, and without thermocycling equipment.

The polymerase chain reaction (PCR) is widely recognized as the most powerful strategy for DNA amplification, and has become an indispensable technique for researchers in virtually every branch of biological research. At the same time, PCR is somewhat limited by the need for expensive and specialized equipment, and there remains a demand for a simplified techniques that allow investigators to perform DNA amplification quickly and cheaply, and in any research setting.

Huimin Kong and his colleagues at New England Biolabs (Beverly, MA) sought to develop an alternative method that emulates the process by which DNA replication takes place in the living cell. *In vivo*, replication is assisted by the action of enzymes known as helicases, which unwind the double helix and separate the two strands; single-strand binding proteins (SSBs) then associate with the unwound DNA, keeping the strands separated so that DNA polymerase can bind and synthesize daughter strands.

With this in mind, Kong's team developed a technique that they call helicase-dependent amplification (HDA), relying on the action of helicases and SSBs as an alternative to heat denaturation for the separation of template strands. Once the DNA molecule has been thusly melted, the amplification primers can bind and polymerase-mediated amplification can proceed. This process allows multiple cycles of replication to be performed at a single incubation temperature, completely eliminating the need for thermocycling equipment.

In initial experiments, Kong's group
heat-denatured a template plasmid, then added *E. coli* UvrD helicase and its

accessory protein MutL, along with SSBs and ATP, and incubated the mixture for one hour at 37°C. The amplification was strong and easily detected on an agarose gel; individual experiments confirmed that each component of the reaction mixture was essential to amplification. In subsequent experiments with DNA from the pathogen *Treponema denticola*, the investigators determined that HDA was capable of achieving more than a ten million-fold amplification of template sequence. It also became apparent that initial heat denaturation improves the efficiency of amplification, but is not a necessary step.

In an effort to test the diagnostic capabilities of HDA, the researchers used the technique to detect B. *malayi* genomic DNA within human blood. In initial assays, they achieved a level of sensitivity capable of detecting 500 pathogen genome copies. Kong indicates that since publication, his group has increased the sensitivity to a point where HDA can detect as few as 100 copies, although he still hopes to be able to improve the system to a threshhold sensitivity of 10 copies. One possibility for improving the robustness and sensitivity of HDA lies in the identification of optimal sources for DNA helicase and polymerase, and Kong says that his group has already improved the assay considerably by experimenting with different enzyme combinations. As of the initial publication, the size limit for efficient amplification was merely 400-bp; since then, refinement of HDA has increased this limit to 1-kb, and the team remains confident that they will continue to expand the limits of this assay.

At present, Kong sees a number of possibilities for HDA as a diagnostic application for point-of-care use, but is very clear that he sees HDA as a complement to rather than a replacement for existing amplification techniques. "I think it's important for people to know that HDA is not going to replace PCR... that's a great technology that has a lot of advantages. I think HDA will offer an alternative to PCR where doing thermocycling is prohibitive. If you're doing molecular diagnostics in poor countries that can't afford a $2,000 thermocycler, you can perhaps do HDA for a couple of dollars without instruments. In these areas... we can still do DNA amplification which will achieve one million- or one billion-fold amplification, so I think it's a good technology."

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- Top

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- Top

Close window